THE EFFECTS OF OCEAN ACIDIFICATION ON MOLT RATE AND CARAPACE AREA IN JUVENILE DUNGENESS CRABS (*Cancer magister*)

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TABLE OF CONTENTS

ABSTRACT

Ocean acidification, the consequence of atmospheric CO_2 dissolving in the ocean to form carbonic acid, is rapidly intensifying. A wealth of marine wildlife is known to be vulnerable to the expected drop in pH, including a number of crab species. The Dungeness crab (*Cancer magister*), the target of a commercially important fishery in the Pacific Northwest, is known to be sensitive to elevated CO_2 levels. In an effort to better characterize the degree to which *C. magister* is sensitive to ocean acidification, this study examined the carapace areas, the distance between the eyes, and the molt rates of juveniles reared for over three hundred days in ambient (low) and future (high) CO_2 conditions across six instars. Statistical analysis indicates that carapace areas and eye distances were significantly smaller in crabs reared in high-CO_2 relative to crabs reared in low-CO_2. Effects on carapace area were detectable at the third through the sixth instar, and effects on eye distance were detectable at the second, third, and fifth instar. Molt rates were significantly slower for crabs in high-CO_2 treatments for the periods of J1 to J2, J2 to J3, and J3 to J4. If dissolved CO_2 levels continue to rise in the ocean, wild juveniles Dungeness crabs may be smaller at each stage and display delays in development. These sensitivities have the potential to affect the crabs' role in US West Coast food webs and their recruitment into fisheries.

CHAPTER 1

INTRODUCTION

Our oceans are experiencing a negative byproduct of escalating anthropogenic carbon dioxide (CO_2) levels in the atmosphere. The consequence of CO_2 dissolving in the ocean is the reaction with water and carbonate ions to produce carbonic acid, which reduces the pH level of seawater. This changing ocean chemistry is the catalyst for ocean acidification (OA), a process that has the potential to introduce a multitude of new osmoregulatory and developmental challenges to marine organisms, especially crustaceans[1,2]. While OA might not kill individual organisms, it has been shown to result in potentially sublethal effects[3].

One predicted outcome of a reduced pH is the decline of the oceanic saturation state of calcium carbonate as a result of elevated levels of dissolved CO_2[3,4]. While not directly fatal, this could cause calcification rates to drop[3] or induce partial carapace dissolution[4] in organisms that require calcium carbonate for shell growth. OA has been shown to affect metabolism and acid-base regulation in marine organisms, disrupting homeostasis and potentially inducing negative physiological trade-offs to compensate for harmful health-related issues[3,5], such as hypercapnia, or concentrated levels of CO_2 in the bloodstream. Body size has also shown to be sensitive to OA as red king crabs (Lithodidae: *Paralithodes camtschaticus*) and Tanner crabs (Oregoniidae: *Chionoecetes bairdi*) both grew slower in high-CO_2 conditions[6]. Behavioral changes have been observed as well, as the mud crab (Panopeidae: *Panopeus herbstii*) displayed foraging impairments after exposure to high-CO_2 conditions[7]. These findings indicate that OA can induce a variety of responses from marine organisms and that sensitivities are not necessarily tied to one taxon.

The aim of this study is to characterize the responses of juvenile Dungeness crabs (Cancridae: *Cancer magister*) to ocean acidification, focusing on variations in carapace size and developmental rates during early juvenile stages. The Dungeness crab fishery yielded nearly $200 million for the US economy in 2013[8]. Changes in the size, shape, or development of this crab could lead to a decline in fishery productivity which might negatively impact a crucial part of the economy in the Pacific Coast of the United States[8,9]. Additionally, *C. magister* has immense cultural importance to the Native American community near Puget Sound and is an important link in the Pacific Northwest ecosystem, serving as both predator and prey in different parts of its life cycle[10].

Juvenile *C. magister* individuals exposed for approximately 30 days to a high-CO_2 treatment responded with elevated ATP production[11]. Additionally, juveniles raised in a compound high-CO_2 and hypoxic treatment showed more severe changes in the generation of metabolites and quantity of amino acids than in solely low pH conditions[11]. These findings suggest that crabs in habitats that are acidified and hypoxic, conditions not uncommon for Puget Sound, might be at a higher risk for maladaptive physiological changes. *C. magister* individuals have also demonstrated sensitivity to OA in larval stages, with delays in molting and decreased survival rates occurring after the larval crabs were reared in a high-CO_2 environment[12].

Considering that *C. magister* has displayed a variety of sensitivities to exposure in acidified seawater, here we examine the degree to which carapace sizes and molt rate (or the number of days between molts) are affected by high-CO_2 conditions. Since the

Industrial Revolution, the average oceanic pH level has dropped by 0.1 units and is projected to continue to decline[13]. In our study area of Puget Sound, Washington, USA, the average pH ranges from 8.1 to 7.6 while future CO_2 conditions are predicted to drop the pH to a range of 7.8 to 7.3, potentially even dipping below 7.1 in some regions of the Sound[13]. We reared juvenile *C. magister* for over 300 days in high and low CO_2 treatments and photo imaged the carapace through the first six juvenile instars, or molt stages. From these data, we analyzed the progression of carapace area and the distance between the eyes and examined the molt rates of juveniles in low- and high-CO_2 conditions. Our findings can inform predictions about future conservation challenges these crabs may face in the wild, as well as predicate the potential economic and ecological ramifications resulting from OA-induced changes in the rate and morphology of their development.

CHAPTER 2

LITERATURE REVIEW

Ocean acidification is an expanding area of scientific investigation, and the more research and modeling that is done to discover its potential reach and impact on marine ecosystems, the more scientists are realizing how detrimental the rise of dissolved CO_2 levels in the ocean can be[3,14,15,16]. The loss of ecologically, economically, and culturally important organisms could have severe negative public and environmental implications and, as a result, these organisms are of particular interest in OA research. An abundance of research is being conducted to identify and characterize potential effects of OA on crabs, especially *Cancer magister*, a critical species for the US West Coast[1,4,5,11,12]. As research into ocean acidification has only recently started gaining momentum, there are several areas where the literature is lacking in-depth investigation. Many of the organismal studies have not exposed marine organisms to high-CO_2 conditions in the laboratory for a prolonged time period (> 30–60 days at a time). Additionally, few experiments studying sensitivities of crabs to OA have analyzed the changes in size or molt rates as a result of exposure to high-CO_2 conditions, especially across early juvenile stages.

The current findings from laboratory experiments with crabs should help inform directions for future experiments, especially as there is a limited depth to the types of species and parameters that have been researched thus far. Long et al. investigated potential physiological and size effects from OA on red king crabs (*Paralithodes camtschaticus*) and Tanner crabs (*Chionoecetes bairdi*)[6], both of which are found naturally in the North Pacific. While the two crab species did not show differences in sizes after OA exposure, they were observed to grow at slower rates than rates observed in crabs reared in ambient treatments. Additionally, red king crabs exhibited a constant condition index (the ratio of dry mass to carapace width or length) and a lower calcium content while Tanner crabs exhibited the exact inverse response, implying that different species of crabs may experience different physiological responses to OA. An experiment by De la Haye et al. suggested that a species of hermit crab (Paguridae: *Pagurus bernhardus*) displayed several behavioral abnormalities after exposure to high-CO_2 conditions[17]. The hermit crabs were less likely to move from a less optimal shell to a more optimal shell and exhibited reduced activity and resource detection under high-CO_2 conditions. Furthermore, a study by Dodd et al. found that mud crabs (Panopeidae: *Panopeus herbstii*) have impaired foraging behaviors, particularly in terms of altered prey handling time and length of unsuccessful predation attempts, after exposure to high-CO_2 conditions[7]. These findings shed light on the potential behavioral and interspecific effects of ocean acidification.

One of the most robust compilations and analyses of early studies conducted on crustaceans in OA was carried out by Whiteley[18]. Whiteley pointed to several different physiological mechanisms in crustaceans that might be influenced by OA, including osmoregulation, acid-base regulation, energy allocation, and calcification/growth rates, which could inform potential future avenues of inquiry. However, these mechanisms might not necessarily be relevant with specific regards to *C. magister*, as Whiteley's study covered a wide range of crustaceans, so more research would be required in order to pinpoint what physiological changes might be underlying physical changes in *C. magister* resulting from elevated CO_2 levels. Widdicombe and Spicer also synthesized preexisting data on the effects of elevated CO_2 levels on bottom-dwelling species, emphasizing how OA will more likely cause sub-lethal rather than lethal effects and how the longevity and adaptability of a species might depend on its physical size[19].

Much of the modeling that predicts the effects of OA on ocean chemistry and organismal physiology accounts for the combination of other environmental elements, such as latitude, hypoxia, and temperature. OA has been found to have a greater negative impact on regions, including effects on ocean chemistry and carbonate saturation levels, among other potential organismal effects, where it is compounded with higher latitudes and hypoxia[3]. Individuals from a species of spider crab (Oregoniidae: *Hyas araneus*) displayed greater physiological and developmental sensitivities to OA and elevated temperatures when they were collected from a region of higher latitude[20]. Moreover, results from a metabolomic analysis showed that juveniles of *C. magister* displayed the most severe physiological reactions (highest change in glycerophospholipid synthesis and metabolite abundance) to combined hypoxic and acidified conditions, as opposed to separate or control treatments[11]. For crabs exposed to high-CO_2 treatments, there was a trend of upregulation in the citric acid cycle and therefore an increase in ATP production.

The physiology and life history of *C. magister* is well documented, particularly in terms of ecological and interspecific interactions between these crabs and other species[10,21,22,23]. Gutermuth and Armstrong contextualized the physiological response of juvenile *C. magister* to changing temperatures, finding that smaller crabs have higher metabolic rates than larger crabs when exposed to high temperatures[24]. Larval *C. magister* exposed to high-CO_2 conditions experienced hatching delays as well as reduced survival rates and developmental delays in the zoaea[12]. This experiment helps to inform my own because it sets a precedent for the sensitivities these crabs have to OA. However, because the larvae were only exposed for their earliest life stage, these findings offer only a limited glimpse into more long-term effects on the crabs' physiology and life history traits. Furthermore, a recent *in-situ* experiment analyzing carapace dissolution in larval *C. magister* discovered that dissolution is highest among crabs exposed to high-CO_2 conditions in coastal waters and that dissolution is negatively related to carapace width[4]. The results from this experiment show the vulnerability of crabs in coastal regions to the effects of ocean acidification.

The study presented here analyzes the responses of juvenile *C. magister* to OA by comparing carapace areas, eye distances, and molt rates of crabs reared in high and low CO_2 treatments for the first six molt stages. I aim to better understand sensitivities experienced by the juveniles after over 300 days in high and low CO_2 treatments. By utilizing ImageJ and R programming, I will be able to detect the degree of change in carapace sizes and if high-CO_2 conditions cause molting rate delays in juveniles. Quantifying how OA affects juvenile *C. magister* in the laboratory will make it easier to predict how these animals might respond in the wild, particularly in regions where they are significant to the people and the ecosystem.

CHAPTER 3

METHODS & MATERIALS

Laboratory System

The test subjects were caught by the Lummi Tribe as megalopae (final larval stage) using light traps from sites at Hale's Pass and Sand Point along Puget Sound, Washington, USA, on 17 August 2018. The megalopae were housed in separate well-plates inside chambers circulating the appropriate treatment CO_2 content. Upon molting into juveniles, the crabs were partitioned into six groups, each in 890-liter tanks: three with high-CO_2 water and three with low-CO_2 water. Seawater directly from Puget Sound was filtered and pumped into the tanks. Initial sample sizes were n = 59 juveniles in the low-CO_2 treatments (with 13, 23, and 23 in each replicate) and n = 79 juveniles in the high-CO_2 treatments (with 21, 28, and 30 in each replicate). Every week the tanks were cleaned, and the crabs were rotated between the treatment tanks to avoid potential tank bias.

The juveniles were housed in cylindrical mesh cups with a fastened lid and sand on the bottom. These cups allowed for water to flow around the crabs and were suspended on a rack near the surface of the water. The cups were kept close together and rearranged weekly as the crabs were rotated between treatment tanks (to eliminate the possibility of position-in-tank bias). Crabs were fed size-appropriate pieces of California squid (*Doryteuthis opalescens*), and occasionally Coho salmon (*Oncorhynchus kisutch*) and geoduck clams (*Panopea generosa*), twice a week.

Treatment Chemistry

The pH levels selected for this experiment are based on current and predicted CO_2 levels for Puget Sound[7]. The low (current) CO_2 treatment was maintained at pH of 7.8 and the high (predicted) CO_2 treatment was maintained at a pH of 7.2. Honeywell UDA2182 sensors were used to control these treatment levels by feeding the appropriate CO_2 concentrations into the tank water and monitoring the salinity levels (maintained at an average of 29.4 ppt for the duration of the experiment). The pH was monitored with weekly spectroscopy measurements in all treatment tanks using Ocean Optics Miniature Spectroscopy software (5 mM solution of Sigma Aldritch m-cresol purple indicator dye).

Analysis of Molt Rate

The crabs were checked daily to record any molts or deaths. If a crab had molted, the molt was extracted from the cup and stored in a separate well-plate. A crab would be photographed within a week after it had molted. Dead crabs were removed from the tanks. The dates of each molt were recorded in a database that was used to find molting rates across instars and to analyze trends between treatments and across stages using both a Repeated Measures Analysis with a mixed model (with random effects for crab identity and stage and fixed effects for treatment) to test for overall differences between treatments and to account for the same individuals being measured multiple times throughout the experiment. An ANOVA model comparison was also run to test the significance of the treatment's effect in the mixed model. A post-hoc linear model was then used to test for treatment differences within stages. These tests were run on the software, R[25] (v.1.2.1335) with the packages nlme, lme4, tidyr, and ggplot2.

Analysis of Carapace Area and Eye Distance

Photographs of the crabs were taken using an Infinity1 microscope camera with Infinity Analyze and Capture software by Lumenera. The images were then imported into ImageJ (FIJI version 1.52e) and the distances were scaled according to known measurements already marked onto the images. The distance between the eyes and the area of the carapace were measured for each crab from the first through the sixth instar (**Fig. 1**). Because the spines outlining the sides of the carapace were often hard to detect because of inconsistent picture resolution, the area is defined as the space of the carapace excluding eye sockets and spines to maintain consistency across treatments and instars. The R packages nlme, lme4, tidyr, and ggplot2 were used to run a Repeated Measures Analysis with a mixed model and a linear model to determine if there was a significant treatment effect on carapace area and eye distance.

CHAPTER 4

RESULTS

After rearing juvenile crabs in high- and low-CO_2 treatments from August 2018 to July 2019, we found a significant effect of CO_2 treatment on molt rate (p=0.0142). With the ANOVA model comparison test, we reaffirmed the treatment to have a significant effect on the data (X^2=0.01331). In particular, we found that individuals reared in high-CO_2 conditions had a significantly longer period between molts for the first (J1) to the second (J2) instar (p=0.0161, F=5.93), J2 to J3 (p<0.001, F=12.97), and J3 to J4 (p=0.00268, F=9.46) (**Fig. 2**). However, there was no significant difference between CO_2 treatments found for the periods of J4 to J5 (p=0.121, F=2.45) or J5 to J6 (p=0.334, F=0.946). In addition to molt rate, a significant overall effect of CO_2 treatment on carapace area was detected (p<0.001, X^2<0.001). Carapace areas for crabs in high-CO_2 conditions were significantly smaller for instars J3 (p=0.0125, F=6.34), J4 (p<0.001, F=30.06), J5 (p<0.001, F=14.03), and J6 (p=0.0281, F=5.13), but not for J1 (p=0.7022, F=1.82) or J2 (p=0.1796, F=1.82) (**Fig. 3**). Lastly, we found a significant overall effect of CO_2 treatment on eye distance (p=0.00151, X^2=0.001522). High-CO_2 crabs had significantly shorter distances between their eyes in instars J2 (p<0.001, F=12.53), J3 (p=0.0219, F=5.40), and J5 (p=0.00465, F=8.43) (**Fig. 4**). However, no significant differences between treatments were detected in the instars J1 (p=0.499, F=0.459), J4 (p=0.0669, F=3.43), or J6 (p=0.636, F=0.227).

CHAPTER 5

FIGURES

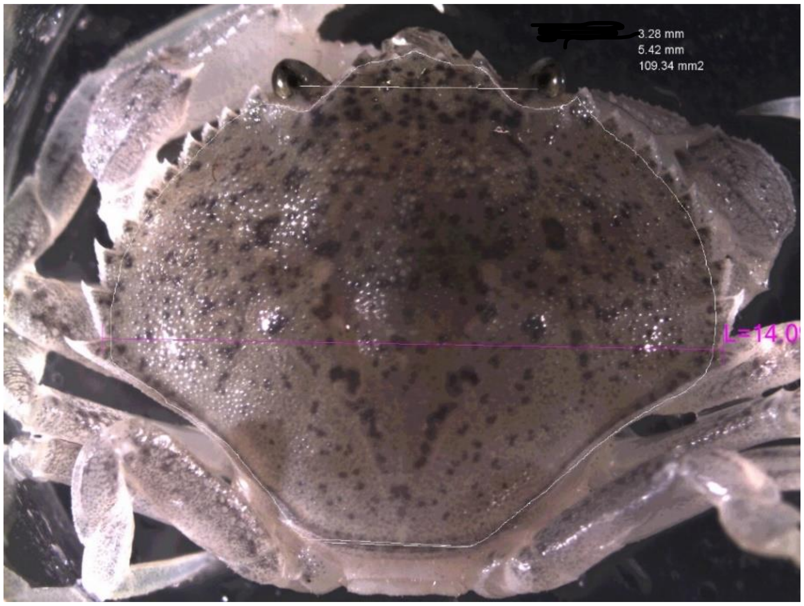

Figure 1. This picture demonstrates the measurements that were taken from each picture once in ImageJ. This crab was in stage J4 and reared in a low-CO_2 treatment, and the line between each of its eyes measured its eye distance (here, found to be 5.42 mm) and the line tracing the circumference of the carapace measured its carapace area (109.34 mm^2). The transverse pink line across the midsection of the crab was marked on the image when the picture was taken and was used to calibrate the length measurements when imported into ImageJ.

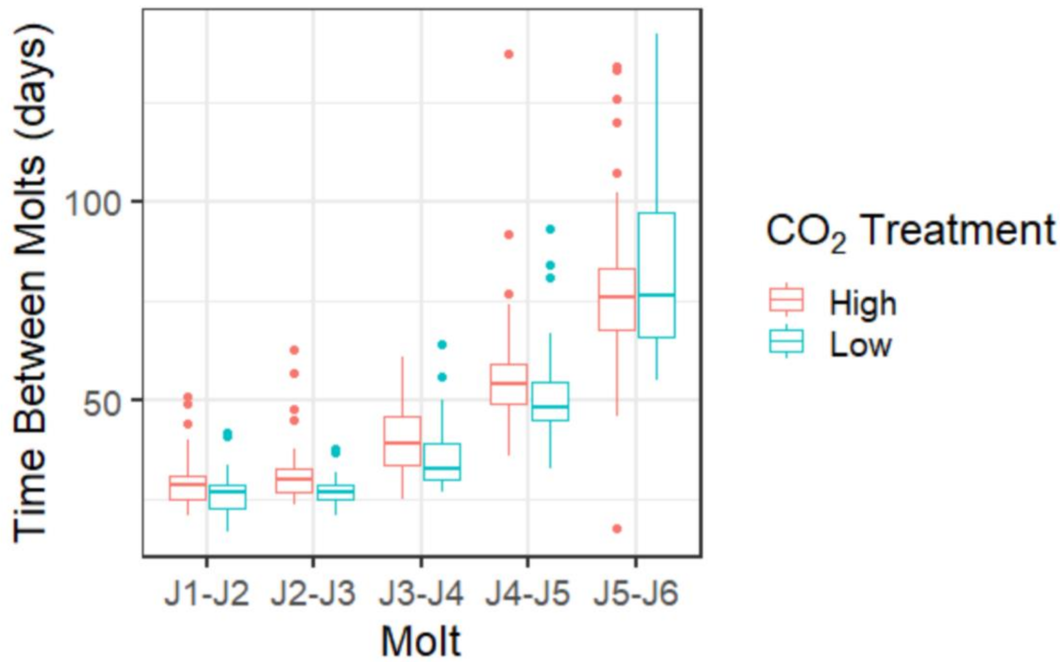

Figure 2. A collection of boxplots showing the means and range in the number of days the crabs in each instar spent in that instar, i.e. the molt rate. The x-axis shows the instar period between molts that was measured, and the y-axis marks the number of days spent in that instar. A significant effect of CO_2 treatment on molt rate was found ($p=0.0142$, $X^2=0.01331$). Molting periods were significantly longer for high-CO_2 crabs in the instars of J1 to J2 ($p=0.0161$, $F=5.93$), J2 to J3 ($p<0.001$, $F=12.97$), and J3 to J4 ($p=0.00268$, $F=9.46$), but were not significantly different for J4 to J5 ($p=0.121$, $F=2.45$) or J5 to J6 ($p=0.334$, $F=0.946$).

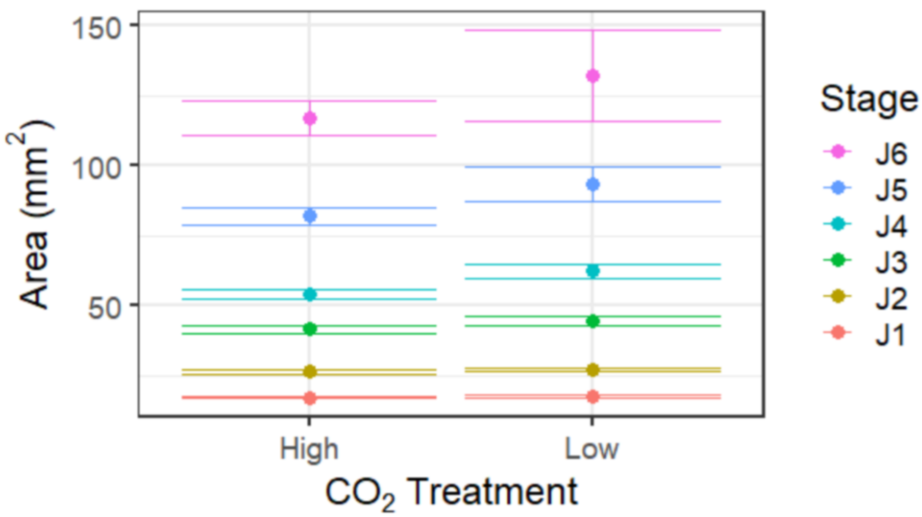

Figure 3. A treatment comparison of carapace areas, showing the 95% confidence intervals for each treatment through the first six juvenile instars. There was a significant treatment effect shown overall between carapace areas for crabs reared in high-CO_2 and low-CO_2 conditions ($p<0.001$, $X^2<0.001$). Carapace areas were significantly smaller for crabs in high-CO_2 treatments for instars J3 ($p=0.0125$, $F=6.34$), J4 ($p<0.001$, $F=30.06$), J5 ($p<0.001$, $F=14.03$), and J6 ($p=0.0281$, $F=5.13$), but showed no significant differences in J1 ($p=0.7022$, $F=1.82$) or J2 ($p=0.1796$, $F=1.82$).

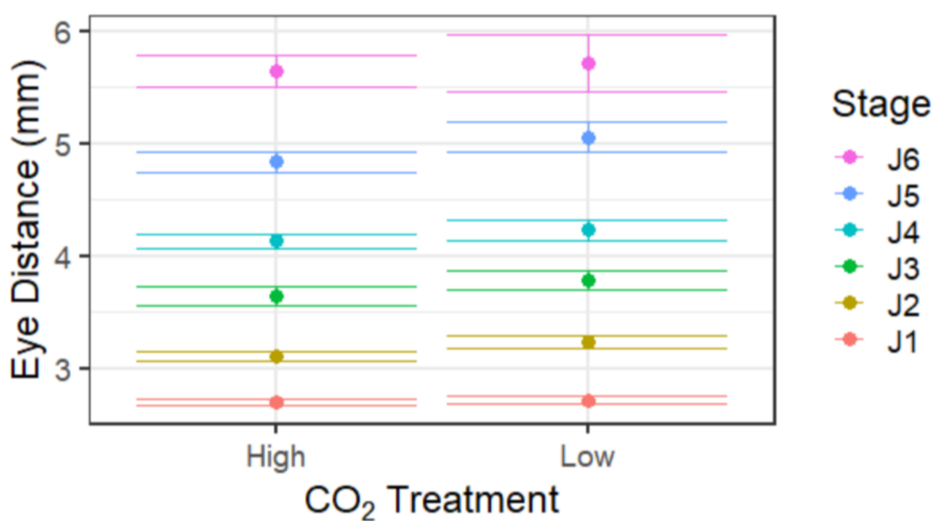

Figure 4. A treatment comparison of the distance between eyes, showing the 95% confidence intervals for each treatment through the first six juvenile instars. There was a

significant treatment effect shown overall between eye distances for crabs reared in high-CO_2 and low-CO_2 conditions ($p=0.00151$, $X^2=0.001522$). Eye distances were significantly shorter for crabs in high-CO_2 treatments in instars J2 ($p<0.001$, $F=12.53$), J3 ($p=0.0219$, $F=5.40$), and J5 ($p=0.00465$, $F=8.43$), but were not significantly different in instars J1 ($p=0.499$, $F=0.459$), J4 ($p=0.0669$, $F=3.43$), or J6 ($p=0.636$, $F=0.227$).

CHAPTER 6

DISCUSSION

The findings presented here align with our hypothesis that crabs reared in high-CO_2 conditions would have slower molt rates than crabs reared in low-CO_2 conditions as the molt periods of J1 to J2, J2 to J3, and J3 to J4 were found to have significantly more days for crabs in high-CO_2 treatments. It is unclear what drove the lack of significant difference in molt rate for instars J4 to J5 and J5 to J6, so further experimentation testing more long-term effects of high-CO_2 exposure could help elucidate this finding. However, one limitation to this study was the relatively small initial sample sizes for each treatment (59 for low-CO_2, 79 for high-CO_2), as that limited the number of crabs that survived or molted to the later instars before the study was concluded.

Our results also showed an effect of CO_2 levels on carapace area in juvenile crabs, which is a valid metric for overall size of the crabs, and eye distance. Crabs reared in high-CO_2 conditions tended to have smaller carapace areas and shorter eye distances than crabs reared in low-CO_2 treatments, particularly in the later stages. However, there was an anomaly in the eye distance findings for stages J4 and J6, as those measurements for high-CO_2 crabs were not significantly different from the low-CO_2 crab. Additionally, it is important to note that the effects of high-CO_2 levels on size are likely to accumulate over the organism's lifetime. If a crab is smaller in the earlier stages, because these effects seem to last beyond the first stage in which they were observed, it is reasonable to expect the differences in size to persist over time and therefore for this individual to remain stunted in later stages as well. While evidence of persistent differences between treatments in later instars did not appear for the molt rates, more experimentation testing long-term exposure to high-CO_2 conditions would be beneficial to our understanding of permanent changes to developmental rates induced by ocean acidification.

Should wild crabs in Puget Sound encounter pH levels consistent with those presented here, this study provides evidence that they will likely have smaller carapace sizes. In turn, fisheries that harvest *C. magister* could be negatively impacted economically if there is a decline in yield per crab. What's more, as larger crabs are more resistant to predation[10], a reduction in size could also affect the role of *C. magister* in the Puget Sound, and broader, ecosystem. As the findings from this study suggest a trend for reduced carapace sizes and elongated periods between molts in juvenile *C. magister* after exposure to ocean acidification, it is evident that steps should be taken to protect this species in areas, like Puget Sound[13,16], where OA is projected to become more severe. Results from this study could assist with legislation to protect the economy surrounding the Dungeness crab fishery and the ecosystem of which this crab is a part.

CHAPTER 7

FUTURE DIRECTIONS

Based on the findings from this study, there is compelling evidence that juvenile *C. magister* are at risk for reduced carapace sizes and delayed development in the wild, should dissolved CO_2 levels continue to rise in Puget Sound. We suggest that future studies should rear *C. magister* individuals from zoea until sexual maturity in high-CO_2 conditions to determine what possible physical changes could occur from such a prolonged exposure and if the degree of difference between high- and low-CO_2 crabs becomes more exaggerated, or plateaus, with longer exposure. Moreover, measuring the volume of these crabs after long-term high-CO_2 exposure could assist with assessing potential future reductions in yield in the Dungeness crab fishery. Lastly, to determine if ocean acidification can induce heritable genetic changes or cross-generational effects, it would be beneficial to study a cohort of *C. magister* spawned from a generation of crabs reared in high-CO_2 conditions.

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